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(54) Title: METHODS TO PROVOKE ANTI–CANCER IMMUNE RESPONSES

(57) Abstract

The present invention provides methods for inducing an antigen–specific immune response. In one aspect, an effective amount of an antigenic peptide binding protein (APBP) and a cytotoxic agent are administered to the subject. In another aspect, an effective amount of a first polynucleotide encoding an antigenic peptide binding protein and a second polynucleotide encoding a cytotoxic agent are administered to the subject. In another aspect, the methods described herein also include administering an effective amount of an antigen presenting cell (APC) recruitment factor, a cytokine or a co–stimulatory molecule. In yet another embodiment, the cytotoxic agent and the antigen presenting cell recruitment factor are encoded by one polynucleotide. In yet another aspect, the invention provides a method of adoptive immunotherapy by administering to a subject a population of educated, antigen–specific immune effector cells, with or without an effective amount of a cytokine and/or a co–stimulator molecule.

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METHODS TO PROVOKE ANTI-CANCER IMMUNE RESPONSES

CROSS REFERENCE TO RELATED APPLICATION

This application claims priority under 35 U.S.C. § 119(e) to U.S. Provisional Application No. 60/078,931, filed March 20, 1998, the contents of which are hereby incorporated by reference into the present disclosure.

TECHNICAL FIELD

This invention is in the field of molecular immunology and medicine. In particular, methods of inducing an antigen-specific immune response are provided.

BACKGROUND

In spite of numerous advances in medical research, cancer remains the second leading cause of death in the United States. In the industrialized nations, roughly one in five persons will die of cancer. Traditional modes of clinical care, such as surgical resection, radiotherapy and chemotherapy, have a significant failure rate, especially for solid tumors. Failure occurs either because the initial tumor is unresponsive, or because of recurrence due to regrowth at the original site and/or metastases.

Cellular immunotherapy is emerging as a technologically and intellectually compelling anti-cancer treatment. The generation of an immune response against tumors has been demonstrated in several animal models and has been inferred from reports of spontaneous tumor regression in man (Stotter and Lotze (1990) Cancer Cells 2:44-55). Cytotoxic T-lymphocyte (CTL) responses can be directed against antigens specifically presented by tumor cells, both *in vivo* and *in vitro*, without the need for prior knowledge of the molecular mechanism by which the tumor arose. In animal models, established tumors can be eradicated by

the adoptive transfer of educated T-cells that specifically recognize malignant cells (Beun et al. (1994) Immunol. Today **15**:11-15).

In order to generate specific T-cells, the naïve T cells must be exposed to antigen presenting cells expressing tumor-specific antigens. The antigens expressed on APCs, such as dendritic cells and macrophages, induce the T-cells to become antigen-specific.

Heat shock proteins (HSPs) may participate in antigen presentation by binding to antigenic peptides. HSPs are synthesized in response to stressful conditions (*e.g.*, heat) and are found in most eukaryotic cellular compartments, for instance HSP70 is found in cytosol while BiP is found in the endoplasmic reticulum. HSPs assist in folding and membrane translocation of nascent proteins, degradation of misfolded proteins and other regulatory processes. They exhibit controlled ATP binding and release of hydrophobic domains and prevent aggregation of nascent chains on polysomes induced by the hydrophobic effect. In addition, HSPs present misfolded proteins of ubiquitination and proteasome-based degradation.

It has recently been shown that heat shock proteins have the ability to render tumor cells more immunogenic. (Lukacs et al. (1997) Gene Therapy **4**:346). Suto et al. (1995) Science **269**:1585-1588 report that heat shock protein:peptide complexes purified from tumor cells and presented to macrophages *in vitro* can cause presentation of tumor antigens.

Vaccines made of peptides bound to carrier proteins (*e.g.*, heat shock proteins) have been proposed as a possible cancer therapy. (Srivastava et al. (1994) Current Opinion in Immun. **6**:728-732; Srivastava (1993) Advances in Cancer Research **62**:153-177; and Tamura et al. (1997) Science **278**:117-120). In theory, the carrier:peptide complexes can be purified from patient tumor biopsy material and then injected back into the same patient in the hope that the complexes will be taken up by the patient's own antigen presenting cells (APCs) and that the antigenic peptides will be released and presented by MHC molecules on the surface of the patient's own APCs, thereby provoking an anti-tumor cell immune response. However, this process is time and labor intensive because

custom tumor-specific vaccines must be generated for each individual patient's tumor. This invention overcomes the failures of prior art therapies and enhances methods for inducing immune responses.

DISCLOSURE OF THE INVENTION

The present invention provides a method for provoking antigen-specific immune responses, and in particular, immune responses against tumor antigens.

In one aspect, the invention provides a method for inducing an antigen-specific immune response, and in one aspect thereby supress the growth of neoplastic or tumor cells, in a subject by administering an effective amount of an antigenic peptide binding protein (APBP) and a cytotoxic agent to the subject. Preferably, the cytotoxic agent is further characterized as being activated by an activating agent upon administration of the activating agent to the subject. The antigenic peptide binding protein may be a heat shock protein (HSP), a soluble major histocompatibility complex (MHC) class I molecule or an antibody engineered to bind antigenic peptides. In one embodiment, the cytotoxic agent is herpes simplex virus thymidine kinase (HSV-tk) and the activating compound is gancyclovir.

In another aspect, the invention provides a method for inducing an antigen-specific immune response in a subject by administering an effective amount of a first polynucleotide encoding an antigenic peptide binding protein and a second polynucleotide encoding a cytotoxic agent. The cytotoxic agent may be further characterized as being activated by an activating agent when it is administered to the subject. The polynucleotides may be naked DNA or may be administered in one or more gene delivery vehicles, for example a retroviral vector, an adenoviral vector, an adeno-associated virus vector and/or a liposome. For this aspect of the invention, the antigenic peptide binding protein may be a heat shock protein (HSP), a soluble major histocompatibility complex (MHC) class I molecule or an antibody engineered to bind antigen peptides. In one embodiment, the cytotoxic agent is herpes simplex virus thymidine kinase (HSV-

tk) and the activating compound is gancyclovir, wherein the first and second polynucleotides are naked DNA.

In another aspect, the methods described herein also include administering an effective amount an antigen presenting cell (APC) recruitment factor. The APC recruitment factor may be interleukin 4 (IL-4), granulocyte- macrophage colony-stimulating factor (GM-CSF), Sepragel and/or macrophage inflammatory protein 3 alpha (MIP3α). In one embodiment, the APC recruitment factor is administered in a gene delivery vehicle. The antigenic peptide binding protein may be a heat shock protein (HSP), a soluble major histocompatibility complex (MHC) class I molecule and/or an antibody engineered to bind antigen peptides. In one embodiment, the cytotoxic agent is herpes simplex virus thymidine kinase (HSV-tk) and the activating agent is gancyclovir.

In a further embodiment, the antigenic peptide binding protein and the cytotoxic agent are encoded by one polynucleotide. In a further embodiment, the antigenic peptide binding protein, the cytotoxic agent and the antigen presenting cell recruitment factor are encoded by one polynucleotide. In another embodiment, the antigenic peptide binding protein and either the cytotoxic agent or the antigen presenting cell recruitment factor are encoded by one polynucleotide. In yet another embodiment, the cytotoxic agent and the antigen presenting cell recruitment factor are encoded by one polynucleotide.

In yet another aspect, the invention provides a method of adoptive immunotherapy by administering to a subject a population of educated, antigen-specific immune effector cells. The cells are made by culturing naïve immune effector cells with antigen presenting cells that have previously been exposed to antigenic peptide binding protein:peptide complexes derived from lysates of tumor cells which in turn, have been genetically modified to express an antigenic peptide binding protein either with or without a cytotoxic gene product. After modification and education, the educated immune effector cells are readministered to the subject. The cytotoxic agent is further characterized by

being activated by an activating agent when the tumor cells are cultured with the activating agent.

As will become apparent, preferred features and characteristics of one aspect of the invention are applicable to any other aspect of the invention.

MODES FOR CARRYING OUT THE INVENTION

Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, "Molecular Cloning: A Laboratory Manual", second edition (Sambrook et al., 1989); "Oligonucleotide Synthesis" (M.J. Gait, ed., 1984); "Animal Cell Culture" (R.I. Freshney, ed., 1987); the series "Methods in Enzymology" (Academic Press, Inc.); "Handbook of Experimental Immunology" (D.M. Weir & C.C. Blackwell, eds.); "Gene Transfer Vectors for Mammalian Cells" (J.M. Miller & M.P. Calos, eds., 1987); "Current Protocols in Molecular Biology" (F.M. Ausubel et al., eds., 1987, and periodic updates); "PCR: The Polymerase Chain Reaction", (Mullis et al., eds., 1994); "Current Protocols in Immunology" (J.E. Coligan et al., eds., 1991).

Definitions

As used herein, certain terms may have the following defined meanings.

As used in the specification and claims, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

The term "immune effector cells" refers to cells that specifically recognize an antigen present, for example on a neoplastic or tumor cell. For the purposes of this invention, immune effector cells include, but are not limited to, B cells, monocytes, macrophages, NK cells and T cells such as cytotoxic T lymphocytes (CTLs), for example CTL lines, CTL clones, and CTLs from tumor, inflammatory, or other infiltrates. "T-lymphocytes" denotes lymphocytes that are phenotypically CD3+, typically detected using an anti-CD3 monoclonal antibody in combination with a suitable labeling technique. The T-lymphocytes of this invention are also generally positive for CD4, CD8, or both. The term "naïve" immune effector cells refers to immune effector cells that have not encountered antigen and is intended to by synonymous with unprimed and virgin. "Educated" refers to immune effector cells that have interacted with an antigen such that they differentiate into an antigen-specific cell.

An "effective amount" is an amount sufficient to effect beneficial or desired results. An effective amount can be administered in one or more administrations, applications or dosages. The polynucleotides of the present invention may be administered or applied transdermally, orally, subcutaneously, intramuscularly, intravenously or parenterally. For purposes of this invention, an effective amount of the polynucleotides is that amount which provokes an antigen-specific immune response in the subject.

The terms "polynucleotide" and "nucleic acid molecule" are used interchangeably to refer to polymeric forms of nucleotides of any length. The polynucleotides may contain deoxyribonucleotides, ribonucleotides, and/or their analogs. Nucleotides may have any three-dimensional structure, and may perform any function, known or unknown. The term "polynucleotide" includes single-, double-stranded and triple helical molecules.

"Oligonucleotide" refers to polynucleotides of between about 5 and about 100 nucleotides of single- or double-stranded DNA. Oligonucleotides are also known as oligomers or oligos and may be isolated from genes, or chemically synthesized by methods known in the art. A "primer" refers to an

oligonucleotide, usually single-stranded, that provides a 3'-hydroxyl end for the initiation of enzyme-mediated nucleic acid synthesis.

The following are non-limiting embodiments of polynucleotides: a gene or gene fragment, exons, introns, mRNA, tRNA, rRNA, ribozymes, cDNA, recombinant polynucleotides, branched polynucleotides, plasmids, vectors, isolated DNA of any sequence, isolated RNA of any sequence, nucleic acid probes, and primers. A nucleic acid molecule may also comprise modified nucleic acid molecules, such as methylated nucleic acid molecules and nucleic acid molecule analogs. Analogs of purines and pyrimidines are known in the art, and include, but are not limited to, aziridinycytosine, 4-acetylcytosine, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethyl-aminomethyluracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, pseudouracil, 5-pentylnyluracil and 2,6-diaminopurine. The use of uracil as a substitute for thymine in a deoxyribonucleic acid is also considered an analogous form of pyrimidine. By way of example only and not to limit this invention, the polynucleotides encode a peptide, a ribozyme or an antisense sequence.

The terms "protein", "oligopeptide", "polypeptide" and "peptide" are used interchangeably to refer to polymers of amino acids of any length. The polymer may be linear or branched, it may comprise modified amino acids, and it may be interrupted by non-amino acids. The terms also encompass an amino acid polymer that has been modified naturally or by intervention; for example, disulfide bond formation, glycosylation, lipidation, acetylation, phosphorylation, or any other manipulation or modification, such as conjugation with a labeling component. Also included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including, for example, unnatural amino acids, etc.), as well as other modification known in the art.

The term "culturing" refers to the *in vitro* propagation of cells or organisms on or in media of various kinds. It is understood that the descendants

of a cell grown in culture may not be completely identical (either morphologically, genetically, or phenotypically) to the parent cell. By "expanded" is meant any proliferation or division of cells.

A "subject" is a vertebrate, preferably a mammal, more preferably a human. Mammals include, but are not limited to, murines, simians, humans, farm animals, sport animals, and pets.

An "antigenic peptide binding protein" is any protein which is capable of binding peptides, preferably peptides that elicit an antigenic response. For example, heat shock proteins and major histocompatibility complex (MHC) molecules have a binding groove that enables them to bind to peptides sequences. The terms "major histocompatibility complex" or "MHC" refers to a complex of genes encoding cell-surface molecules that are required for antigen presentation to T cells and for rapid graft rejection. In humans, the MHC complex is also known as the HLA complex. The proteins encoded by the MHC complex are known as "MHC molecules" and are classified into class I and class II MHC molecules. Class I MHC molecules include membrane heterodimeric proteins made up of an α chain encoded in the MHC associated noncovalently with β2-microglobulin. Class I MHC molecules are expressed by nearly all nucleated cells and have been shown to function in antigen presentation to $CD8^+$ T cells. Class I molecules include HLA-A, -B, and -C in humans. Class II MHC molecules also include membrane heterodimeric proteins consisting of noncovalently associated α and β chains. Class II MHC are known to function in $CD4^+$ T cells and, in humans, include HLA-DP, -DQ, and DR.

The term "heat shock protein (HSP)" refers the class of proteins present in most animals which are encoded for by genes that are transcribed suddenly, quickly, and with coordination when the animal is exposed to certain types of stress such as a sudden temperature increase. Non-limiting examples of HSP are HSP gp96, HSP90, HSP70, HSP65, HSP28 and the like. These are commercially available from StressGen Biotechnologies, Victoria, Canada. Polynucleotide sequences encoding these proteins are known in the Art.

A "cytotoxic agent" includes proteins and other molecules which are toxic to a cell, for example by disrupting the cell's normal function. The cytotoxic agent may be a molecule which is itself toxic to the cell or it may be "conditionally activated" in that it generates a cytotoxic agent when provided with an "activating compound", *i.e.* pro-drug. An example of a conditionally cytotoxic agent is herpes simplex virus thymidine kinase (HSV-tk) which is activated by the pro-drug gancyclovir.

The terms "antigen presenting cell recruitment factors" or "APC recruitment factors" includes both intact, whole cells as well as other molecules which are capable of recruiting antigen presenting cells. Examples of suitable APC recruitment factors include molecules such as interleukin 4 (IL4), granulocyte macrophage colony stimulating factor (GM-CSF), Sepragel and macrophage inflammatory protein 3 alpha (MIP3α). These are available from Genzyme (Framingham, MA), Immunex, Schering-Plough and R&D Systems (Minneapolis, MN). They also can be recombinantly produced using the methods disclosed in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F.M. Ausubel et al., eds., (1987)). Peptides, proteins and compounds having the same biological activity as the above-noted factors are included within the scope of this invention.

For purposes of the present invention, it is contemplated that a single protein could possess two or more of the three functions described above, *i.e.* antigen presenting cell recruitment, cytotoxic agents and/or antigenic peptide binding. This multifunctional molecule may be encoded for by a naturally occurring gene or, alternatively, may be engineered using recombinant techniques.

"Co-stimulatory molecules" are involved in the interaction between receptor-ligand pairs expressed on the surface of antigen presenting cells and T cells. Research accumulated over the past several years has demonstrated convincingly that resting T cells require at least two signals for induction of cytokine gene expression and proliferation (Schwartz, R.H. (1990) Science **248**:1349-1356; Jenkins M.K. (1992) Immunol. Today **13**:69-73). One signal, the one that confers specificity, can be produced by interaction of the TCR/CD3

complex with an appropriate MHC/peptide complex. The second signal is not antigen specific and is termed the "co-stimulatory" signal. This signal was originally defined as an activity provided by bone-marrow-derived accessory cells such as macrophages and dendritic cells, the so called "professional" APCs. Several molecules have been shown to enhance co-stimulatory activity. These are heat stable antigen (HSA) (Liu Y. et al. (1992) J. Exp. Med. 175:437-445); chondroitin sulfate-modified MHC invariant chain (Ii-CS) (Naujokas M.F. et al. (1993) Cell 74:257-268); intracellular adhesion molecule 1 (ICAM-1) (Van Seventer G.A. (1990) J. Immunol. **144**:4579-4586); B7-1, and B7-2/B70 (Schwartz R.H. (1992) Cell 71:1065-1068). These molecules each appear to assist co-stimulation by interacting with their cognate ligands on the T cells. Co-stimulatory molecules mediate co-stimulatory signal(s) which are necessary, under normal physiological conditions, to achieve full activation of naïve T cells. One exemplary receptor-ligand pair is the B7 co-stimulatory molecule on the surface of APCs and its counter-receptor CD28 or CTLA-4 on T cells (Freeman et al. (1993) Science **262**:909-911; Young et al. (1992) J. Clin. Invest. **90**:229; and Nabavi et al. (1992) Nature **360**:266-268). Other important co-stimulatory molecules are CD40, CD54, CD80, CD86. The term "co-stimulatory molecule" encompasses any single molecule or combination of molecules which, when acting together with a peptide/MHC complex bound by a TCR on the surface of a T cell, provides a co-stimulatory effect which achieves activation of the T cell that binds the peptide. The term thus encompasses B7, or other co-stimulatory molecule(s) on an antigen-presenting matrix such as an APC, fragments thereof (alone, complexed with another molecule(s), or as part of a fusion protein) which, together with peptide/MHC complex, binds to a cognate ligand and results in activation of the T cell when the TCR on the surface of the T cell specifically binds the peptide. Co-stimulatory molecules are commercially available from a variety of sources, including, for example, Beckman Coulter. It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified co-stimulatory molecules (e.g., recombinantly

produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

As used herein, the term "cytokine" refers to any one of the numerous factors that exert a variety of effects on cells, for example, inducing growth or proliferation. Non-limiting examples of cytokines which may be used alone or in combination in the practice of the present invention include, interleukin-2 (IL-2), stem cell factor (SCF), interleukin 3 (IL-3), interleukin 6 (IL-6), interleukin 12 (IL-12), G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF), interleukin-1 alpha (IL-1α), interleukin-11 (IL-11), MIP-1α, leukemia inhibitory factor (LIF), c-kit ligand, thrombopoietin (TPO) and flt3 ligand. The present invention also includes culture conditions in which one or more cytokine is specifically excluded from the medium. Cytokines are commercially available from several vendors such as, for example, Genzyme (Framingham, MA), Genentech (South San Francisco, CA), Amgen (Thousand Oaks, CA), R&D Systems and Immunex (Seattle, WA). It is intended, although not always explicitly stated, that molecules having similar biological activity as wild-type or purified cytokines (e.g., recombinantly produced or muteins thereof) are intended to be used within the spirit and scope of the invention.

As used herein, "expression" refers to the process by which polynucleotides are transcribed into mRNA and translated into peptides, polypeptides, or proteins. If the polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA, if an appropriate eukaryotic host is selected. Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the *lac* promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG (Sambrook et al. (1989) *supra*). Similarly, an eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors can be obtained commercially or assembled by the sequences described in methods well

known in the art, for example, the methods described below for constructing vectors in general.

"Host cell" is intended to include any individual cell or cell culture which can be or have been recipients for vectors or the incorporation of exogenous nucleic acid molecules, polynucleotides and/or proteins. It also is intended to include progeny of a single cell, and the progeny may not necessarily be completely identical (in morphology or in genomic or total DNA complement) to the original parent cell due to natural, accidental, or deliberate mutation. The cells may be procaryotic or eucaryotic, and include but are not limited to bacterial cells, yeast cells, animal cells, and mammalian cells, e.g., murine, rat, simian or human.

A "gene delivery vehicle" is defined as any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, viruses, such as baculovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

A"viral vector" is defined as a recombinantly produced virus or viral particle that comprises a polynucleotide to be delivered into a host cell, either *in vivo*, *ex vivo* or *in vitro*. Examples of viral vectors include retroviral vectors, adenovirus vectors, adeno-associated virus vectors and the like. In aspects where gene transfer is mediated by a retroviral vector, a vector construct refers to the polynucleotide comprising the retroviral genome or part thereof, and a therapeutic gene. Non-viral gene delivery vehicles include DNA/liposome complexes, and targeted viral protein DNA complexes. To enhance delivery to a cell, the nucleic acid or proteins of this invention can be conjugated to antibodies or binding fragments thereof which bind cell surface antigens, e.g., TCR, CD3 or CD4. Liposomes that also comprise a targeting antibody or fragment thereof can be used in the methods of this invention. This invention also provides the targeting complexes for use in the methods disclosed herein.

The term "immune effector molecule", as used herein, refers to molecules capable of antigen-specific binding, and includes antibodies, T cell antigen receptors, and MHC Class I and Class II molecules.

A "naïve" immune effector cell is an immune effector cell that has never been exposed to an antigen.

As used herein, the term "educated, antigen-specific immune effector cell", is an immune effector cell as defined above, which has encountered antigen and which is specific for that antigen. An educated, antigen-specific immune effector cell may be activated upon binding antigen. "Activated" implies that the cell is no longer in G_0 phase, and begins to produce cytokines characteristic of the cell type. For example, activated CD4+ T cells secrete IL-2 and have a higher number of high affinity IL-2 receptors on their cell surfaces relative to resting CD4+ T cells.

The term "preferentially recognized" intends that a polypeptide of the invention is substantially not recognized, as defined above, by a T cell specific for an unrelated antigen. Assays for determining whether an epitope is recognized by an antigen-specific T cell are known in the art and are described herein.

As used herein, the terms "neoplastic cells", "neoplasia", "tumor", "tumor cells", "cancer" and "cancer cells", (used interchangeably) refer to cells which exhibit relatively autonomous growth, so that they exhibit an aberrant growth phenotype characterized by a significant loss of control of cell proliferation (i.e., de-regulated cell division). Neoplastic cells can be malignant or benign.

"Suppressing" tumor growth indicates a growth state that is curtailed when compared to growth without contact with educated, antigen-specific immune effector cells described herein. Tumor cell growth can be assessed by any means known in the art, including, but not limited to, measuring tumor size, determining whether tumor cells are proliferating using a ^{3}H-thymidine incorporation assay, or counting tumor cells. "Suppressing" tumor cell growth means any or all of the following states: slowing, delaying, and stopping tumor growth, as well as tumor shrinkage.

The term "culturing" refers to the *in vitro* propagation of cells or organisms on or in media of various kinds. It is understood that the descendants of a cell grown in culture may not be completely identical (morphologically, genetically, or phenotypically) to the parent cell. By "expanded" is meant any proliferation or division of cells.

A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro*, *in vivo* or *ex vivo*.

As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

Materials and Methods

In one aspect, the present invention provides a method of inducing an antigen-specific immune response in a subject by administering to that subject an effective amount of an antigenic peptide binding protein (APBP) and a cytotoxic agent. The APBP and cytotoxic agent are described in more detail below. The APBP and cytotoxic agent can be administered to the subject as molecules, *e.g.*, proteins or, alternatively, as polynucleotides encoding these compounds which can be introduced into the subject *via* a gene delivery vehicle. In another aspect, at least one of the APBP or the cytotoxic agent is administered as a whole molecule while the other is administered via a polynucleotide inserted into a gene delivery vehicle.

In another aspect, an antigen-specific immune response is induced in a subject by administering an effective amount of an APBP, a cytotoxic agent and an effective amount of antigen presenting cell (APC) recruitment factor. In this aspect, the APBP, cytotoxic agent and APC recruitment factor can be administered as whole molecules; *via* polynucleotides encoding these molecules; or as combinations of whole molecules and polynucleotides. Suitable APC recruitment factors are discussed herein.

The methods described herein can be practiced *in vivo, ex vivo* or *in vitro*. In particular, the APBP, the cytotoxic agent and the optional APC recruiting factor can be administered directly to a subject as molecules or encoded for by polynucleotides in gene delivery vehicles. Alternatively, *ex vivo* methods involve subjecting cells obtained from the subject to the methods described herein and administering those cells back to the patient. Finally, the methods of the present invention can be used in an *in vitro* cell culture to generate antigen-specific immune effector cells. These cells can then be used as a therapeutic agent.

Thus, the present invention provides methods of inducing an immune response that leads to destruction of distant metastases following locoregional administration of the described molecules to an accessible tumor mass, such as a cutaneous skin lesion or nodule. The antigenic peptides within a cancer cell are loaded onto the abundant APBP and the APBP:peptide complexes are released

from the cancer cell as the cell is destroyed by the action of the cytotoxic agent. The released APBP:peptide complexes are taken up by APCs recruited to the treated site (*e.g.*, by the APC recruitment factor) and this results in presentation of antigenic peptides. Once the peptides have been presented on the cells surface, an anti-tumor cell immune response is mounted and these educated, antigen-specific immune effector cells destroy local and distant cells expressing the tumor-specific antigen. These and other advantages of the present invention are discussed herein.

Antigenic peptide binding proteins

For purposes of the present invention, an antigenic peptide binding protein (APBP) includes any molecule, preferably a peptide, which can bind antigenic peptides. In a preferred embodiment, the APBP is of human origin. Perhaps one of the most well-known naturally occurring APBP are the membrane-bound glycoproteins encoded for by the genes known as the major histocompatibility complex (MHC). These proteins are involved in presenting a wide variety of antigens on the surface of a cell. In the region of the protein farthest away from the cell membrane, a cleft (or groove) binds to an antigenic peptide created by proteolytic activity within the cell. Through a complex series of the processing steps, the antigenic peptide bound to the groove in the MHC is then presented on the surface of the cell, where it can be recognized by the immune system.

In a preferred embodiment, the APBP is a heat shock proteins (HSP), for example HSP65. Like the MHC proteins, the heat shock proteins have a peptide binding groove and are able bind short peptides sequences that are generated by intracellular protein proteolysis mediated by proteosome complexes. Heat shock proteins may also participate in the loading of these bound peptide fragments onto MHC molecules which are subsequently presented at the cell's surface. Thus, HSP:peptide complexes can provide a rich source of antigenic peptides, and complexes purified from a transformed cell can provoke immune responses specific to the transformed cell. Anti-HSP antibody assays can be used as a means to monitor the therapy using methods and commercially available materials from StressGen Biotechnologies, Victoria, Canada. Alternatively, using

techniques well-known in the art and described in Harlow and Lane (1989) *supra*, anti-HSP monoclonal antibodies can be raised and used to monitor the therapy.

Thus, presentation of the antigens such as tumor associated antigens by the APCs elicits a strong immune response resulting in destruction of tumor cells by antigen-specific immune effector cells such as cytotoxic T lymphocyte cells (CTLs). The induction of the CTL response is one method to assay for a positive response to the therapy and a means to confirm the biological activity of new factors useful in the methods of this invention. The presence of a large number of T-cells in tumor has been correlated with a prognostically favorable outcome in some cases (Whiteside and Parmiani (1994) Cancer Immunol. Immunother. **39**:15-21). Woolley et al. (1995) Immmunology **84**:55-63, have shown that implantation of polyurethane sponges containing irradiated tumor cells can efficiently trap anti-tumor CTLs (4-times greater than lymph fluid, 50-times greater than spleen or peripheral blood). Following activation with T-cell cytokines in the presence of their appropriately presented recognition antigen, TILs proliferate in culture and acquire potent anti-tumor cytolytic properties. Weidmann et al. (1994) Cancer Immunol. Immunother. **39**:1-14.

Monitoring of tumor regression *in vivo* or the use of assays to determine T cell response which arewell known in the art can be utilized to determine if the object of the methods described herein have been achieved. For example, any method that will compare T cell number prior to and subsequent to therapy can be utilized. In addition, the induction of co-stimulatory molecules by these methods could also stimulate anergic or low affinity self-reactive CTL clones. Methods to assay for CTL clones include: standard ^{51}Cr release assay as describe in Kawakami et al. (1988) J. Exp. Med. **168**:2183-91. Briefly, cytotoxic T cells are added to target cells previously loaded with ^{51}Cr and one measures the release of ^{51}Cr from the lysed target cells. Cytokine release assay as described in Kawakami et al. (1994) PNAS **91**:3515-19. Briefly, cytotoxic T cells are added to target cells and one measures the amount of IFN γ released by ELISA. To measure the relative proportion of immune effector cells within a mixed population that recognize a particular target the Enzyme-linked immunospot (ELISPOT) assay is

employed as described in Czerkinsky et al. (1988) J. Immunol. Meths. **110**:29-36. Briefly, 96 well nitrocellulose-bottomed plates are coated with an anti-cytokine antibody, generally anti-interferon-γ. Target cells and immune effector cells such as cytotoxic T cells (CTLs) are added to wells. Cytokine released from the CTLs is captured by the anti-interferon-γ antibody and quantitated using a standard ELISA format.

As noted above, the amount of APBP:antigen complexes can also be determined, for example, by generating antibodies against the APBP and using immunological techniques, such as an ELISA assay. Thus, where HSP65 is used as the ABPB, the antibody will be specific for HSP65.

Cytotoxic Agents

The present invention also encompasses the use of a cytotoxic agent to destroy the cancer cell thereby releasing the APBP:peptide complex. Agents that are cytotoxic to cells include any substance that disrupts cellular function, preferably to a sufficient extent to lyse the target cell. In some aspects, the cytotoxic compound may exhibit a bystander effect in that the compound is toxic to both the cells into which it is taken up and, in addition, to neighboring cells.

In one embodiment, the cytotoxic agent is cytotoxic without further modifications. Examples include cytotoxic agents that do not require a prodrug include toxins (e.g., ricin toxin), specific tumor suppressor gene products (e.g., p53), signaling molecules that can trigger cell death (e.g., Fas ligand and TNFα), pro-apoptotic factors (e.g., bcl-xs as in Clarke, et al. (1995) PNAS **92**:1104-11029), anti-sense RNA or ribozymes specific for mRNA encoding proteins vital for cell survival, proteases, DNAses and RNAses.

In a preferred embodiment, the cytotoxic agent is activated by an activating agent. In other words, an activating compound must be administered to generate a cytotoxic agent. For example, herpes simplex virus thymidine kinase (HSV-tk) can be administered to a subject, either as a peptide or through a gene delivery vehicle carrying a polynucleotide encoding the peptide. However, HSV-tk does not become cytotoxic until an activating compound is added, for instance

gancyclovir or acyclovir. (Available from Hoffman-LaRoche, Nutley, New Jersey.) Other examples of cytotoxic agents that do require a prodrug include *E.coli* cytosine deaminase, *E. coli* purine nucleoside phosphorylase, *E. coli* nitroreductase, mammalian cytochrome p450 isozymes, carboxypeptidase G2 and mammalian thymidine phosphorylase.

Because one purpose of the cytotoxic agent is to release antigen-specific APBP:peptide complexes from a tumor cell at the time when these complexes are maximally concentrated, it is desirable to control when the cytotoxic agent will destroy the cell. In one embodiment, this is achieved by controlling the temporal expression of the cytotoxic agent and peptide binding protein genes or by delivering the cytotoxic compound (or gene encoding the compound) to the cancer cells at a time after the delivery of the APBP. Alternatively, a conditionally active cytotoxic gene that encodes a protein that is cytotoxic in itself when supplemented with a cofactor or activating component or that is able to generate a cytotoxic compound when provided with an appropriate cofactor or prodrug may be administered and activated at the appropriate time.

In one embodiment, the polynucleotides encoding the cytotoxic agent and the APBP are delivered in separate gene delivery vehicles. Here, it is unlikely that all the cancer cells that express one polynucleotide will express the other. Therefore, to maximize destruction of those cells containing high intracellular levels of APBP:peptide complexes, it is desirable for the cytotoxic agent to have a significant bystander effect, *i.e.* lyse cells neighboring the cells into which the gene delivery vehicle was successfully introduced and transcribed. The need for a cytotoxic agent having a significant bystander effect is greatly reduced in the embodiment where the polynucleotides encoding the cytotoxic agent and the APBP are delivered in the same gene delivery vehicle.

Antigen Presenting Cell (APC) Recruitment Factors

Antigen presentation cells (APC) are cells which are capable of inducing the presentation of one or more antigens, preferably with class I MHC molecules. APCs can be intact, whole cells as well as other molecules, for example,

macrophages, dendritic cells, B cells, purified MHC class I molecules complexed to β2-microglobulin, hybrid APCs and foster antigen presenting cells.

Dendritic cells (DCs) are believed to be the most potent antigen-presenting cells (APCs). It has been shown that DCs provide all the signals required for T cell activation and proliferation. These signals can be categorized into two types. The first type, which gives specificity to the immune response, is mediated through interaction between the T-cell receptor/CD3 ("TCR/CD3") complex and an antigenic peptide presented by a major histocompatibility complex ("MHC") class I or II protein on the surface of APCs. This interaction is necessary, but not sufficient, for T cell activation to occur. In fact, without the second type of signals, the first type of signals can result in T cell anergy. The second type of signals, called co-stimulatory signals, is neither antigen-specific nor MHC-restricted, and can lead to a full proliferation response of T cells and induction of T cell effector functions in the presence of the first type of signals.

In one embodiment of the present invention, one or more factor(s) that recruit antigen presenting cells are also administered. The APC recruitment factor ensures that a high localized concentration of antigen presenting cells is available in the vicinity of the disrupted cancer cells to take up the liberated APBP:peptide complexes. The APCs recruited to the site have the ability to process the available APBP:peptide complexes in the context of MHC molecules, thereby inducing an anti-cancer immune response through education of immune effector cells. In a further embodiment, an effective amount of a cytokine and/or co-stimulatory molecules is administered in the form of a protein or polynucleotide encoding the protein, to enhance the immune response.

Gene Delivery Vehicles

In one aspect, polynucleotides encoding one or more of the APBP, cytotoxic agent or APC recruitment factor can be delivered to the subject using a gene delivery vehicle. The methods of this invention are intended to encompass any method of gene transfer into the subject. Examples of delivery mechanisms

include, but are not limited to viral mediated gene transfer, liposome mediated transfer, transformation, transfection and transduction, *e.g.*, viral mediated gene transfer such as the use of vectors based on DNA viruses such as adenovirus, adeno-associated virus and herpes virus, as well as retroviral based vectors.

Vectors Useful in Genetic Modifications

In general, genetic modifications of cells *in vitro, ex vivo* and *in vivo*, employed in the present invention are accomplished by introducing a vector containing a polypeptide or transgene encoding a heterologous or an altered antigen. A variety of different gene transfer vectors, including viral as well as non-viral systems can be used. Viral vectors useful in the genetic modifications of this invention include, but are not limited to adenovirus, adeno-associated virus vectors, retroviral vectors and adeno-retroviral chimeric vectors.

Construction of Recombinant Adenoviral Vectors or Adeno-Associated Virus Vectors

Adenovirus and adeno-associated virus vectors useful in the genetic modifications of this invention may be produced according to methods already taught in the art. (see, *e.g.*, Karlsson et al. (1986) EMBO 5:2377; Carter (1992) Current Opinion in Biotechnology 3:533-539; and Muzcyzka (1992) Current Top. Microbiol. Immunol. **158**:97-129; GENE TARGETING: A PRACTICAL APPROACH (1992) ed. A. L. Joyner, Oxford University Press, NY). Several different approaches are feasible. Preferred is the helper-independent replication deficient human adenovirus system.

The recombinant adenoviral vectors based on the human adenovirus 5 (Virology **163**:614-617, 1988) are missing essential early genes from the adenoviral genome (usually E1A/E1B), and are therefore unable to replicate unless grown in permissive cell lines that provide the missing gene products *in trans*. In place of the missing adenoviral genomic sequences, a transgene of interest can be cloned and expressed in cells infected with the replication deficient adenovirus. Although adenovirus-based gene transfer does not result in

integration of the transgene into the host genome (less than 0.1% adenovirus-mediated transfections result in transgene incorporation into host DNA), and therefore is not stable, adenoviral vectors can be propagated in high titer and transfect non-replicating cells. Human 293 cells, which are human embryonic kidney cells transformed with adenovirus E1A/E1B genes, typify useful permissive cell lines and are commercially available from the ATCC (Manassas, VA). However, other cell lines which allow replication-deficient adenoviral vectors to propagate therein can be used, including HeLa cells.

Additional references describing adenovirus vectors and other viral vectors which could be used in the methods of the present invention include the following: Horwitz M.S., Adenoviridae and Their Replication, in Fields, B., et al. (eds.) VIROLOGY, Vol. 2, Raven Press New York, pp. 1679-1721, 1990); Graham F. et al. pp. 109-128 in METHODS IN MOLECULAR BIOLOGY, Vol. 7: GENE TRANSFER AND EXPRESSION PROTOCOLS, Murray, E. (ed.), Humana Press, Clifton, N.J. (1991); Miller N. et al. (1995) FASEB Journal **9**:190-199; Schreier H (1994) Pharmaceutica Acta Helvetiae **68**:145-159; Schneider and French (1993) Circulation **88**:1937-1942; Curiel D.T. et al. (1992) Human Gene Therapy **3**:147-154; Graham F.L. et al. WO 95/00655; Falck-Pedersen E.S. WO 95/16772; Denefle P. et al. WO 95/23867; Haddada H. et al. WO 94/26914; Perricaudet M. et al. WO 95/02697; and Zhang W. et al. WO 95/25071. A variety of adenovirus plasmids are also available from commercial sources, including, e.g., Microbix Biosystems of Toronto, Ontario (see, e.g., Microbix Product Information Sheet: Plasmids for Adenovirus Vector Construction, 1996). See also, the papers by Vile et al. (1997) Nature Biotechnology **15**:840-841 and Feng, et al.(1997) Nature Biotechnology, **15**:866-870, describing the construction and use of adeno-retroviral chimeric vectors that can be employed for genetic modifications.

Additional references describing AAV vectors which could be used in the methods of the present invention include the following: Carter B. HANDBOOK OF PARVOVIRUSES, Vol. **I**, pp. 169-228, 1990; Berns, VIROLOGY, pp. 1743-1764 (Raven Press 1990); Carter B. (1992) Curr. Opin. Biotechnol. **3**:533-539; Muzyczka N. (1992) Current Topics in Micro and Immunol. **158**:92-129; Flotte

T.R. et al. (1992) Am. J. Respir. Cell Mol. Biol. 7:349-356; Chatterjee et al. (1995) Ann. NY Acad. Sci. **770**:79-90; Flotte T.R. et al. WO 95/13365; Trempe J.P. et al., WO 95/13392; Kotin R. (1994) Human Gene Therapy, **5**:793-801, 1994; Flotte T.R. et al. (1995) Gene Therapy **2**:357-362; Allen J.M. WO 96/17947; and Du et al. (1996) Gene Therapy **3**:254-261.

Construction of Retroviral Vectors

Retroviral vectors useful in the methods of this invention are produced recombinantly by procedures already taught in the art. For example, WO 94/29438 describes the construction of retroviral packaging plasmids and packaging cell lines. As is apparent to the skilled artisan, the retroviral vectors useful in the methods of this invention are capable of infecting the cells described herein. The techniques used to construct vectors, and transfix and infect cells are widely practiced in the art. Examples of retroviral vectors are those derived from murine, avian or primate retroviruses. Retroviral vectors based on the Moloney murine leukemia virus (MoMLV) are the most commonly used because of the availability of retroviral variants that efficiently infect human cells. Other suitable vectors include those based on the Gibbon Ape Leukemia Virus (GALV) or HIV.

In producing retroviral vector constructs derived from the Moloney murine leukemia virus (MoMLV), in most cases, the viral gag, pol and env sequences are removed from the virus, creating room for insertion of foreign DNA sequences. Genes encoded by the foreign DNA are usually expressed under the control of the strong viral promoter in the LTR. Such a construct can be packed into viral particles efficiently if the gag, pol and env functions are provided *in trans* by a packaging cell line. Thus, when the vector construct is introduced into the packaging cell, the gag-pol and env proteins produced by the cell, assemble with the vector RNA to produce infectious virions that are secreted into the culture medium. The virus thus produced can infect and integrate into the DNA of the target cell, but does not produce infectious viral particles since it is lacking essential packaging sequences. Most of the packaging cell lines currently in use have been transfected with separate plasmids, each containing one of the

necessary coding sequences, so that multiple recombination events are necessary before a replication competent virus can be produced. Alternatively, the packaging cell line harbors an integrated provirus. The provirus has been crippled so that, although it produces all the proteins required to assemble infectious viruses, its own RNA cannot be packaged into virus. Instead, RNA produced from the recombinant virus is packaged. The virus stock released from the packaging cells thus contains only recombinant virus.

The range of host cells that may be infected by a retrovirus or retroviral vector is determined by the viral envelope protein. The recombinant virus can be used to infect virtually any other cell type recognized by the env protein provided by the packaging cell, resulting in the integration of the viral genome in the transduced cell and the stable production of the foreign gene product. In general, murine ecotropic env of MoMLV allows infection of rodent cells, whereas amphotropic env allows infection of rodent, avian and some primate cells, including human cells. Amphotropic packaging cell lines for use with MoMLV systems are known in the art and commercially available and include, but are not limited to, PA12 and PA317. Miller, et al. (1985) Mol. Cell. Biol. 5:431-437; Miller, et al. (1986) Mol. Cell. Biol. 6:2895-2902; and Danos, et al. (1988) Proc. Natl. Acad. Sci. USA 85:6460-6464. Xenotropic vector systems exist which also allow infection of human cells.

The host range of retroviral vectors has been altered by substituting the env protein of the base virus with that of a second virus. The resulting, "pseudotyped", virus has the host range of the virus donating the envelope protein and expressed by the packaging cell line. Recently, the G-glycoprotein from vesicular stomatitis virus (VSV-G) has been substituted for the MoMLV env protein. Burns, et al. (1993) Proc. Natl. Acad. Sci USA 90:8033-8037; and PCT WO 92/14829. Since infection is not dependent on a specific receptor, VSV-G pseudotyped vectors have a broad host range.

Usually, the vectors will contain at least two heterologous genes or gene sequences: (i) the therapeutic gene to be transferred; and (ii) a marker gene that enables tracking of infected cells. As used herein, "therapeutic gene" can be an

entire gene or only the functionally active fragment of the gene capable of compensating for the deficiency in the patient that arises from the defective endogenous gene. Therapeutic gene also encompasses antisense oligonucleotides or genes useful for antisense suppression and ribozymes for ribozyme-mediated therapy. For example, in the present invention, a therapeutic gene may be one that neutralizes the immunosuppressive factor or counter its effects.

Therapeutic genes that encode dominant inhibitory oligonucleotides and peptides as well as genes that encode regulatory proteins and oligonucleotides also are encompassed by this invention. Generally, gene therapy will involve the transfer of a single therapeutic gene although more than one gene may be necessary for the treatment of particular diseases. In one embodiment, the therapeutic gene is a dominant inhibiting mutant of the wild-type immunosuppressive agent. Alternatively, the therapeutic gene could be a wild-type, copy of a defective gene or a functional homologue.

More than one gene can be administered per vector or alternatively, more than one gene can be delivered using several compatible vectors. Depending on the genetic defect, the therapeutic gene can include the regulatory and untranslated sequences. For gene therapy in human patients, the therapeutic gene will generally be of human origin although genes from other closely related species that exhibit high homology and biologically identical or equivalent function in humans may be used, if the gene product does not induce an adverse immune reaction in the recipient. The therapeutic gene suitable for use in treatment will vary with the disease.

Nucleotide sequences for the therapeutic gene will generally be known in the art or can be obtained from various sequence databases such as GenBank. The therapeutic gene itself will generally be available or can be isolated and cloned using the polymerase chain reaction PCR (Perkin-Elmer) and other standard recombinant techniques. The skilled artisan will readily recognize that any therapeutic gene can be excised as a compatible restriction fragment and placed in a vector in such a manner as to allow proper expression of the therapeutic gene in hematopoietic cells.

A marker gene can be included in the vector for the purpose of monitoring successful transduction and for selection of cells into which the DNA has been integrated, as against cells which have not integrated the DNA construct. Various marker genes include, but are not limited to, antibiotic resistance markers, such as resistance to G418 or hygromycin. Less conveniently, negative selection may be used, including, but not limited to, where the marker is the HSV-tk gene, which will make the cells sensitive to agents such as acyclovir and gancyclovir. Alternatively, selections could be accomplished by employment of a stable cell surface marker to select for transgene expressing cells by FACS sorting. The NeoR (neomycin /G418 resistance) gene is commonly used but any convenient marker gene whose sequences are not already present in the recipient cell, can be used.

The viral vector can be modified to incorporate chimeric envelope proteins or nonviral membrane proteins into retroviral particles to improve particle stability and expand the host range or to permit cell type-specific targeting during infection. The production of retroviral vectors that have altered host range is taught, for example, in WO 92/14829 and WO 93/14188. Retroviral vectors that can target specific cell types *in vivo* are also taught, for example, in Kasahara et al. (1994) Science **266**:1373-1376. Kasahara, et al. describe the construction of a Moloney leukemia virus (MoMLV) having a chimeric envelope protein consisting of human erythropoietin (EPO) fused with the viral envelope protein. This hybrid virus shows tissue tropism for human red blood progenitor cells that bear the receptor for EPO, and is therefore useful in gene therapy of sickle cell anemia and thalassemia. Retroviral vectors capable of specifically targeting infection of cells are preferred for *in vivo* gene therapy.

The viral constructs can be prepared in a variety of conventional ways. Numerous vectors are now available which provide the desired features, such as long terminal repeats, marker genes, and restriction sites, which may be further modified by techniques known in the art. The constructs may encode a signal peptide sequence to ensure that cell surface or secreted proteins encoded by genes are properly processed post-translationally and expressed on the cell surface if

appropriate. Preferably, the foreign gene(s) is under the control of a cell specific promoter.

Expression of the transferred gene can be controlled in a variety of ways depending on the purpose of gene transfer and the desired effect. Thus, the introduced gene may be put under the control of a promoter that will cause the gene to be expressed constitutively, only under specific physiologic conditions, or in particular cell types.

The retroviral LTR (long terminal repeat) is active in most hematopoietic cells *in vivo* and will generally be relied upon for transcription of the inserted sequences and their constitutive expression (Ohashi et al. (1992) Proc. Natl. Acad. Sci. **89**:11332; and Correll, et al. (1992) Blood **80**:331). Other suitable promoters include the human cytomegalovirus (CMV) immediate early promoter and the U3 region promoter of the Moloney Murine Sarcoma Virus (MMSV), Rous Sarcoma Virus (RSV) or Spleen Focus Forming Virus (SFFV).

Examples of promoters that may be used to cause expression of the introduced sequence in specific cell types include Granzyme A for expression in T-cells and NK cells, the CD34 promoter for expression in stem and progenitor cells, the CD8 promoter for expression in cytotoxic T-cells, and the CD11b promoter for expression in myeloid cells.

Inducible promoters may be used for gene expression under certain physiologic conditions. For example, an electrophile response element may be used to induce expression of a chemoresistance gene in response to electrophilic molecules. The therapeutic benefit may be further increased by targeting the gene product to the appropriate cellular location, for example the nucleus, by attaching the appropriate localizing sequences.

The vector construct is introduced into a packaging cell line which will generate infectious virions. Packaging cell lines capable of generating high titers of replication-defective recombinant viruses are known in the art, see for example, WO 94/29438. Viral particles are harvested from the cell supernatant and purified for *in vivo* infection using methods known in the art such as by filtration of supernatants 48 hours post transfection. The viral titer is determined by infection

of a constant number of appropriate cells (depending on the retrovirus) with titrations of viral supernatants. The transduction efficiency can be assayed 48 hours later by a variety of methods, including Southern blotting.

After viral transduction, the presence of the viral vector in the transduced cells or their progeny can be verified such as by PCR. PCR can be performed to detect the marker gene or other virally transduced sequences. Generally, periodic blood samples are taken and PCR conveniently performed using e.g. NeoR probes if the NeoR gene is used as marker. The presence of virally transduced sequences in bone marrow cells or mature hematopoietic cells is evidence of successful reconstitution by the transduced cells. PCR techniques and reagents are well known in the art, See, generally, PCR PROTOCOLS, A GUIDE TO METHODS AND APPLICATIONS. Innis, Gelfand, Sninsky & White, eds. (Academic Press, Inc., San Diego, 1990) and commercially available (Perkin-Elmer).

Non-viral vectors, such as plasmid vectors useful in the genetic modifications of this invention, can be produced according to methods taught in the art. References describing the construction of non-viral vectors include the following: Ledley FD (1995) Human Gene Therapy **6**:1129-1144; Miller N. et al. (1995) FASEB Journal **9**:190-199; Chonn,A. et al. (1995) Curr. Opin. in Biotech. **6**:698-708; Schofield JP et al. (1995) British Med. Bull. **51**:56-71; Brigham K.L. et al. (1993) J. Liposome Res. **3**:31-49; Brigham K.L. WO 91/06309; Felgner P.L. et al. WO 91/17424; Solodin et al. (1995) Biochemistry **34**:13537-13544; WO 93/19768; Debs et al. WO 93/25673; Felgner P.L. et al.; U.S. Patent 5,264,618; Epand R.M. et al. U.S. Patent 5,283,185; Gebeyehu et al. U.S. Patent 5,334,761; Felgner P.L. et al. U.S. Patent 5,459,127; Overell R.W. et al. WO 95/28494; Jessee WO 95/02698; Haces and Ciccarone, WO 95/17373; and Lin et al. WO 96/01840.

More than one gene can be administered per vector or alternatively, more than one gene can be delivered using several compatible vectors. Depending on the genetic defect, the vector can include the regulatory and untranslated sequences. For gene therapy in human patients, the polynucleotides encoding the APBP and/or APC recruitment factor will generally be of human origin although genes from other closely related species that exhibit high homology and

biologically identical or equivalent function in humans may be used, if the gene product does not induce an adverse immune reaction in the recipient. The therapeutic gene suitable for use in treatment will vary with the disease.

Nucleotide sequences for the polynucleotides will generally be known in the art or can be obtained from various sequence databases such as GenBank. The polynucleotides themselves may also be available or can be isolated and cloned using the polymerase chain reaction PCR (Perkin-Elmer) and other standard recombinant techniques. The skilled artisan will readily recognize that any gene encoding a molecule of interest can be excised as a compatible restriction fragment and placed in a vector in such a manner as to allow proper expression in the subject.

It is known within the state of the art that minor modification to a nucleotide sequence will not affect the function of the molecules encoded thereby. Thus, biologically equivalent polynucleotides of published sequences are also useful in the methods described herein. These polynucleotides can be identified by hybridization under stringent conditions to the sequences disclosed in the published references or known in the art. Alternatively, the polynucleotides can be identified as being at least 80%, or more preferably, at least 90% or most preferably, at least 95%, identical to the disclosed sequences using sequence alignment programs and default parameters.

"Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogstein binding, or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PCR reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

Examples of stringent hybridization conditions include: incubation temperatures of about 25°C to about 37°C; hybridization buffer concentrations of about 6 X SSC to about 10 X SSC; formamide concentrations of about 0% to

about 25%; and wash solutions of about 6 X SSC. Examples of moderate hybridization conditions include: incubation temperatures of about 40°C to about 50°C; buffer concentrations of about 9 X SSC to about 2 X SSC; formamide concentrations of about 30% to about 50%; and wash solutions of about 5 X SSC to about 2 X SSC. Examples of high stringency conditions include: incubation temperatures of about 55°C to about 68°C; buffer concentrations of about 1 X SSC to about 0.1 X SSC; formamide concentrations of about 55% to about 75%; and wash solutions of about 1 X SSC, 0.1 X SSC, or deionized water. In general, hybridization incubation times are from 5 minutes to 24 hours, with 1, 2, or more washing steps, and wash incubation times are about 1, 2, or 15 minutes. SSC is 0.15 M NaCl and 15 mM citrate buffer. It is understood that equivalents of SSC using other buffer systems can be employed.

That a polynucleotide or polynucleotide region (or a polypeptide or polypeptide region) has a certain percentage (for example, 80%, 85%, 90%, or 95%) of "sequence identity" to another sequence means that, when aligned, that percentage of bases (or amino acids) are the same in comparing the two sequences. This alignment and the percent homology or sequence identity can be determined using software programs known in the art, for example those described in CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (F.M. Ausubel et al. eds. (1987)) Supplement 30, section 7.7.18, Table 7.7.1. Preferably, default parameters are used for alignment. A preferred alignment program is BLAST, using default parameters. In particular, preferred programs are BLASTN and BLASTP, using the following default parameters: Genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + SwissProtein + SPupdate + PIR. Details of these programs can be found at the following Internet address: http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST.

***In vivo, Ex vivo* or *In vitro* Introduction of Gene Delivery Vehicles**

The gene delivery vehicles carrying the polynucleotides described herein can be introduced in the host cell *in vivo, ex vivo* or *in vitro*. In one embodiment, the gene delivery vehicles are introduced *in vivo* to a subject. The vehicle may be introduced by transdermal, oral, subcutaneous, intravenous, intramuscular or parenteral modes (Wan, et al. (1997) Human Gene Therapy 8:1355-63). For example, where the tumor to be treated includes a site such as a skin lesion or nodule (*e.g.*, melanoma), the gene delivery vehicle(s) could be applied topically to the skin or injected subcutaneously into the nodule or lesion. Liposomal gene delivery vehicles, for example, can penetrate into the epidermis when topically applied.

In another embodiment, *ex vivo* gene therapy techniques can be employed. Here, the molecules of gene delivery vehicles carrying the genes encoding the molecules are introduced into cells outside of the subject and the transduced cells reintroduced into the subject. Preferably, the cells are obtained from a biopsy sample taken from the subject. The cells can be cultured and a gene delivery vehicle carrying a polynucleotide encoding an APBP introduced into the cells in culture. Optionally, transduction with a cytotoxic agent can also be performed in culture. The cells can then be reintroduced into the subject and the cytotoxic agent activated *in vivo*, causing release of the APBP:peptide complexes in the subject.

In yet another embodiment, the method described herein can be practiced *in vitro*. For example, a tumor biopsy sample can be isolated as described below. In culture, molecules or polynucleotides encoding the APBP, cytotoxic agent and, optionally, APC recruitment factor can then be introduced using one or more gene delivery vehicles. In addition, APCs and naïve immune effector cells should be added -- the APCs will present the tumor-specific antigens and educate the naïve immune cells. The antigen-specific immune effector cells can then be reintroduced in the subject to specifically recognize and destroy the tumor cells.

Isolation of Tumor Cells

Tumor cells can be isolated by any method known in the art. In one embodiment, a biopsy sample is minced and a cell suspension created. Preferably, the tumor cells are separated from other cells (such as immune effector cells, *e.g.*, T cells) using methods well known in the art.

In general, it is desirable to isolate the initial inoculation population from neoplastic cells prior to culture. Separation of the various cell types from neoplastic cells can be performed by any number of methods, including cell sorters, magnetic beads, packed columns. Other procedures for separation can include, but are not limited to, physical separation, magnetic separation, using antibody-coated magnetic beads, affinity chromatography, cytotoxic agents joined to a monoclonal antibody or used in conjunction with a monoclonal antibody, including, but not limited to, complement and cytotoxins, and "panning" with antibody attached to a solid matrix, *e.g.*, plate, elutriation or any other convenient technique.

The use of physical separation techniques include, but are not limited to, those based on differences in physical (density gradient centrifugation and counter-flow centrifugal elutriation), cell surface (lectin and antibody affinity), and vital staining properties (mitochondria-binding dye rho123 and DNA-binding dye Hoechst 33342). These procedures are well known to those of skill in this art.

Monoclonal antibodies are another useful reagent for identifying markers associated with particular cell lineages and/or stages of differentiation can be used. The antibodies can be attached to a solid support to allow for crude separation. The separation techniques employed should maximize the retention of viability of the fraction to be collected. Various techniques of different efficacy can be employed to obtain "relatively crude" separations. Such separations are up to 10%, usually not more than about 5%, preferably not more than about 1%, of the total cells present not having the marker can remain with the cell population to be retained. The particular technique employed will depend upon efficiency of separation, associated cytotoxicity, ease and speed of performance, and necessity for sophisticated equipment and/or technical skill.

Another method of separating cellular fractions is to employ culture conditions which allow for the preferential proliferation of the desired cell populations using methods described in Kawakami et al. (1988) J. Exp. Med. 168:2183-91.

Assessing Efficacy of Gene Transfer *In vitro* or *In vivo*

The efficacy of gene transfer into the cells of the subject can be monitored by any method known in the art. For example, as described above, a reporter or marker gene can be included in the gene delivery vehicle to facilitate identification of those cells into which the vehicle is successfully incorporated (Kass-Eisler, et al. (1994) Gene Therapy 1:395-402). Especially in the *in vitro* and *ex vivo* contexts, marker genes may prove especially helpful. Screening markers or reporter genes are genes that encode a product that can readily be assayed. Non-limiting examples of screening markers include genes encoding for green fluorescent protein (GFP) or genes encoding for a modified fluorescent protein.

Preferably, the marker gene included in the delivery vehicle is a selectable marker. A "positive" selectable marker gene encodes a product that enables only the cells that carry the gene to survive and/or grow under certain conditions. For example, plant and animal cells that express the introduced neomycin resistance (Neo^r) gene are resistant to the compound G418. Cells that do not carry the Neo^r gene marker are killed by G418. Negative selectable marker genes encode a product that allows cells expressing that product to be selectively killed. For example, as described above the conditionally activated cytotoxic agent may also be a selectable marker such as HSV-tk. Cells expressing this gene can be selectively killed using gancyclovir or acyclovir.

Other methods that can be employed to determine the extent of gene transfer include:

(1) quantitation of vector specific DNA by PCR;

(2) quantitation of transgene specific mRNA by RT-PCR;

(3) quantitation of the amount of transgene product in the serum by ELISA if the transgene encodes a soluble, secreted factor such as α1-anti-trypsin;

(4) quantitation of the amount of anti-transgene product antibody in the serum by ELISA; and

(5) quantitation of the amount of transgene product biological activity within the target tissue by homogenization of the tissue and assaying for enzymatic activity or treating the tissue with a suitable substrate that will be converted by the transgene product into a compound that can be quantitated by colorimetric methods.

Isolation, Culturing and Expansion of APCs, Including Dendritic Cells

The following is a brief description of two fundamental approaches for the isolation of APC. These approaches involve (1) isolating bone marrow precursor cells ($CD34^+$) from blood and stimulating them to differentiate into APC; or (2) collecting the precommitted APCs from peripheral blood. In the first approach, the patient must be treated with cytokines such as G-CSF to boost the number of circulating $CD34^+$ stem cells in the peripheral blood.

The second approach for isolating APCs is to collect the relatively rare precommitted APCs already circulating in the blood. Previous techniques for isolating committed APCs from human peripheral blood have involved combinations of physical procedures such as metrizamide gradients and adherence/nonadherence steps (Freudenthal PS et al. (1990) PNAS **87**:7698-7702); Percoll gradient separations (Mehta-Damani et al. (1994) J. Immunol. **153**:996-1003); and fluorescence activated cell sorting techniques (Thomas R. et al. (1993) J. Immunol. **151**:6840-52).

One technique for separating large numbers of cells from one another is known as countercurrent centrifugal elutriation (CCE). In this technique, cells are subject to simultaneous centrifugation and a washout stream of buffer which is constantly increasing in flow rate. The constantly increasing countercurrent flow of buffer leads to fractional cell separations that are largely based on cell size.

In one aspect of the invention, the APC are precommitted or mature dendritic cells which can be isolated from the white blood cell fraction of a mammal, such as a murine, simian or a human (See, *e.g.*, WO 96/23060). The white blood cell fraction can be from the peripheral blood of the mammal. This method includes the following steps: (a) providing a white blood cell fraction obtained from a mammalian source by methods known in the art such as leukophoresis; (b) separating the white blood cell fraction of step (a) into four or more subfractions by countercurrent centrifugal elutriation, (c) stimulating conversion of monocytes in one or more fractions from step (b) to dendritic cells by contacting the cells with calcium ionophore, (d) identifying the dendritic cell-enriched fraction from step (c), and (e) collecting the enriched fraction of step (d), preferably at about 4°C. One way to identify the dendritic cell-enriched fraction is by fluorescence-activated cell sorting. The white blood cell fraction can be treated with calcium ionophore in the presence of other cytokines, such as rhIL-12, rhGM-CSF, or rhIL-4. The cells of the white blood cell fraction can be washed in buffer and suspended in Ca^{++}/Mg^{++} free media prior to the separating step. The white blood cell fraction can be obtained by leukapheresis. The dendritic cells can be identified by the presence of at least one of the following markers: HLA-DR, HLA-DQ, or B7. 2, and the simultaneous absence of the following markers: CD3, CD14, CD16,56,57, and CD 19, 20. Monoclonal antibodies specific to these cell surface markers are commercially available.

More specifically, the method requires collecting an enriched collection of white cells and platelets from leukapheresis that is then further fractionated by countercurrent centrifugal elutriation (CCE) (Abrahamsen TG et al. (1991) J. Clin. Apheresis. 6:48-53). Cell samples are placed in a special elutriation rotor. The rotor is then spun at a constant speed of, for example, 3000 rpm. Once the rotor has reached the desired speed, pressurized air is used to control the flow rate of cells. Cells in the elutriator are subjected to simultaneous centrifugation and a washout stream of buffer which is constantly increasing in flow rate. This results in fractional cell separations based largely but not exclusively on differences in cell size.

Quality control of APC and more specifically DC collection and confirmation of their successful activation in culture is dependent upon a simultaneous multi-color FACS analysis technique which monitors both monocytes and the dendritic cell subpopulation as well as possible contaminant T lymphocytes. It is based upon the fact that DCs do not express the following markers: CD3 (T cell); CD14 (monocyte); CD16, 56, 57 (NK/LAK cells); CD19, 20 (B cells). At the same time, DCs do express large quantities of HLA-DR, significant HLA-DQ and B7.2 (but little or no B7.1) at the time they are circulating in the blood (in addition they express Leu M7 and M9, myeloid markers which are also expressed by monocytes and neutrophils).

In one embodiment, the APCs and cells expressing one or more antigens are autologous. In another embodiment, the APCs and cells expressing the antigen are allogeneic, *i.e.*, derived from a different subject.

Therapeutic Applications

The methods described herein are useful in providing *in vivo, ex vivo* and *in vitro* therapies for diseases associated with antigen-specific immune responses, especially cancers. The APBP and cytotoxic agent can be administered *in vivo* to a tumor site where they act on the tumor cells to release an abundant amount of tumor-specific peptides bound to the APBP. These APBP:peptide complexes are presented by antigen presenting cells, preferably recruited to the site using various recruitment factors. The APCs then serve to educate immune effector cells to specifically bind and destroy the tumor cells throughout the subject.

The methods are also applicable *ex vivo* by isolating tumor cells from a subject, introducing an effective amount of APBP and a conditionally activated cytotoxic agent to these cells, and administering the cells to the subject. When the cytotoxic agent is activated (by administering the appropriate prodrug), the immune-specific response is generated.

The methods described herein can also be used in adoptive immunotherapy regimes. Tumor cells from a biopsy sample are isolated and culture *in vitro*. The APBP, the cytotoxic agent and, optionally the APC

recruitment factor (or polynucleotides encoding these molecules) are then introduced into the culture, for example using one or more gene delivery vehicles. When APCs and naïve immune effector cells are also introduced into the tumor cell culture, the APCs will present the tumor-specific antigens and subsequently educate the naïve immune cells to become tumor-specific. The antigen-specific immune effector cells can then be reintroduced in the subject to specifically recognize and destroy the tumor cells. The antigen-specific immune effector cells can be used for autologous or allogeneic immunotherapy regimes.

The present invention makes use of these APCs to stimulate production of an enriched population of antigen-specific immune effector cells. The antigen-specific immune effector cells are expanded at the expense of the APCs, which die in the culture. The process by which naïve immune effector cells become educated by other cells is described essentially in Coulie (1997) Molec. Med. Today 261-268.

The APCs prepared as described above are mixed with naïve immune effector cells. Preferably, the cells may be cultured in the presence of a cytokine, for example IL2. Because dendritic cells secrete potent immunostimulatory cytokines, such as IL12, it may not be necessary to add supplemental cytokines during the first and successive rounds of expansion. In any event, the culture conditions are such that the antigen-specific immune effector cells expand (*i.e.* proliferate) at a much higher rate than the APCs. Multiple infusions of hybrid cells and optional cytokines can be performed to further expand the population of antigen-specific cells.

The therapy can be applied either *in vivo* or *ex vivo*. For *in vivo* therapy, murine B16 melanoma cells (2×10^4) are injected subcutaneously into the flank of C57BL/6 mice. Approximately 10-14 days later when tumors are 7 mm by 9 mm, 5×10^9 to 1×10^{10} of an adenoviral vector encoding an antigenic peptide binding protein such as a heat shock protein and an APC recruitment factor such as GM-CSF is injected intratumorally. The following day an adenoviral vector encoding a cytotoxic factor such as HSV-TK is injected into the tumor. One day later the prodrug ganciclovir is administered I.P. twice daily to the animals (as described in

O'Malley et al. (1995) Cancer Research 55:1080-5) and every day thereafter for a course of 6 days. Tumor size and survival is monitored as a function of time. Tumor-free animals would be rechallenged with unmodified B16 tumor cells in the contralateral flank to determine if they are protected due to the generation of systemic anti-tumor cell immunity.

For *ex vivo* therapy, murine B16 melanoma cells are infected *in vitro* with an adenovirus encoding an antigenic peptide binding protein (such as an hsp) and APC recruitment factor (such as GM-CSF) and with a second adenovirus encoding a cytotoxic factor (such as HSV TK). The tumor cells are then injected subcutaneously into the flank of C57BL/6 mice. One day later the prodrug ganciclovir is administered I.P. twice daily to the animals (as above) and every day thereafter for a course of 6 days. Tumor size and survival is monitored as a function of time as above and protection from subsequent tumor cell challenge would be assessed. Animals resistant to tumor challenge as a consequence of the therapy would be sacrificed and splenocytes would be harvested and assayed for anti-tumor T cell reactivity.

Example: Melanoma or Head and Neck Therapy

Patients with accessible lesions of melanoma or head and neck cancer patients are injected with a vector or vectors encoding the antigenic peptide binding protein, the cytotoxic factor and an APC recruitment factor. If the cytotoxic factor is HSV TK, the prodrug ganciclovir would be administered systemically via IV infusion. Tumor regression and anti-tumor cell immune responses are monitored.

It is to be understood that while the invention has been described in conjunction with the above embodiments, that the foregoing description and example are intended to illustrate and not limit the scope of the invention. For example, any of the above-noted compositions and/or methods can be combined with known therapies or compositions. Other aspects, advantages and modifications within the scope of the invention will be apparent to those skilled in the art to which the invention pertains.

CLAIMS

1. A method for inducing an antigen-specific immune response in a subject, comprising administering an effective amount of an antigenic peptide binding protein (APBP) and a cytotoxic agent to the subject.

2. The method according to claim 1, wherein cytotoxic agent is further characterized as being activated by an activating agent and the method further comprises administering the activating agent to the subject.

3. The method according to claim 1, wherein the antigenic peptide binding protein is selected from the group consisting of a heat shock protein (HSP), a soluble major histocompatibility complex (MHC) class I molecule and an antibody engineered to bind antigen peptides.

4. The method according to claim 1, wherein the conditionally activated cytotoxic agent is herpes simplex virus thymidine kinase (HSV-tk) and the compound is gancyclovir.

5. A method for inducing an antigen-specific immune response in a subject, comprising administering an effective amount of a first polynucleotide encoding an antigenic peptide binding protein and a second polynucleotide encoding a cytotoxic agent to the subject.

6. The method according to claim 5, wherein cytotoxic agent is further characterized as being activated by an activating agent and the method further comprises administering the activating agent to the subject.

7. The method according to claim 5, wherein the antigenic peptide binding protein is selected from the group consisting of a heat shock protein

(HSP), a soluble major histocompatibility complex (MHC) class I molecule and an antibody engineered to bind antigen peptides.

8. The method according to claim 6, wherein the cytotoxic agent is herpes simplex virus thymidine kinase (HSV-tk) and the activating agent is gancyclovir.

9. The method according to claim 5, wherein the first and second polynucleotides are naked DNA.

10. The method according to claim 5, wherein the first and second polynucleotides are administered in one gene delivery vehicle.

11. The method according to claim 5, wherein the first and second polynucleotides are administered in first and second gene delivery vehicles.

12. The method according to claim 10 wherein the gene delivery vehicle is selected from the group consisting of a retroviral vector, an adenoviral vector, an adeno-associated virus vector and a liposome.

13. The method according to claim 11 wherein the first and second gene delivery vehicles are selected from the group consisting of a retroviral vector, an adenoviral vector, an adeno-associated virus vector and a liposome.

14. The method according to claim 1 or 2 further comprising administering an effective amount an antigen presenting cell (APC) recruitment factor to the subject.

15. The method according to claim 14 wherein the APC recruitment factor is selected from the group consisting of interleukin 4 (IL-4), granulocyte-

macrophage colony-stimulating factor (GM-CSF), Sepragel and macrophage inflammatory protein 3 alpha (MIP3α).

16. The method according to claim 14 wherein the antigenic peptide binding protein is selected from the group consisting of a heat shock protein (HSP), a soluble major histocompatibility complex (MHC) class I molecule and an antibody engineered to bind antigen peptides.

17. The method according to claim 14 wherein the cytotoxic agent is herpes simplex virus thymidine kinase (HSV-tk) and the activating agent is gancyclovir.

18. The method according to claim 5 or 6 further comprising administering an effective amount of an antigen presenting cell (APC) recruitment factor to the subject.

19. The method according to claim 18 wherein the APC recruitment factor is encoded by a third polynucleotide.

20. The method according to claim 18 wherein the APC recruitment factor is selected from the group consisting of interleukin 4 (IL-4), granulocyte-macrophage colony-stimulating factor (GM-CSF), Sepragel and macrophage inflammatory protein 3 alpha (MIP3α).

21. The method according to claim 18 wherein the antigenic peptide binding protein is selected from the group consisting of a heat shock protein (HSP), a soluble major histocompatibility complex (MHC) class I molecule and an antibody engineered to bind antigen peptides.

22. The method according to claim 19 wherein the first, second and third polynucleotides are DNA.

23. The method according to claim 19 wherein the first, second and third polynucleotides are administered in one gene delivery vehicle.

24. The method according to claim 19 wherein the first, second and third polynucleotides are administered in two or more gene delivery vehicles.

25. The method according to claim 23 wherein the gene delivery vehicle is selected from the group consisting of a retroviral vector, an adenoviral vector, an adeno-associated virus vector and a liposome.

26. The method according to claim 24 wherein the two or more gene delivery vehicles are selected from the group consisting of a retroviral vector, an adenoviral vector, an adeno-associated virus vector and a liposome.

27. The method according to claim 5 or 6 wherein the antigenic peptide binding protein and the cytotoxic agent are encoded by one polynucleotide.

28. The method according to claim 18 wherein antigenic peptide binding protein, the cytotoxic agent and the antigen presenting cell recruitment factor are encoded by one polynucleotide.

29. The method according to claim 18 wherein the antigenic peptide binding protein and either the cytotoxic agent or the antigen presenting cell recruitment factor are encoded by one polynucleotide.

30. The method according to claim 18 wherein the cytotoxic agent and the antigen presenting cell recruitment factor are encoded by one polynucleotide.

31. A method of adoptive immunotherapy comprising administering to a subject a population of educated, antigen-specific immune effector cells made by culturing naïve effector cells with antigen presenting cells that have previously been exposed to antigenic peptide binding protein:peptide complexes derived from lysates of tumor cells that have been genetically modified to express an antigenic peptide binding protein either with or without a cytotoxic gene product and reintroducing the educated immune effector cells to the subject.

32. The method according to claim 31 wherein the cytotoxic agent is further characterized by being activated by an activating agent and the method further comprises culturing the tumor cells with the activating agent.

33. The method according to claim 31 wherein the APBP is selected from the group consisting of a heat shock protein (HSP), soluble major histocompatibility complex (MHC) class I molecule and an antibody engineered to bind antigen peptides.

34. The method acccording to claim 32 wherein the cytotoxic agent is herpes simplex virus thymidine kinase (HSV-tk) and the activating agent is gancyclovir.

35. The method according to claim 31 wherein the APBP and cytotoxic agent are encoded for by a first and second polynucleotide.

36. The method according to claim 31, wherein the antigen-specific immune effector cells administered to the subject are autologous.

37. The method according to claim 31, wherein the antigen-specific immune effector cells administered to the subject are allogeneic.

38. The method of any of claims 1, 5, or 31 further comprising administering an effective amount of a cytokine and/or a co-stimulatory molecule to the subject.

39. The method of claim 38, wherein the cytokine an/or co-stimulatory molecule is administered as a polynucleotide coding for the cytokine and/or co-stimulatory molecule.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06048

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/130.1, 138.1, 183.1, 278.1, 93.71; 435/ 325, 355, 372.3, 373; 514/2, 44; 530/350, 391.7; 536/23.1, 23.2, 23.4, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG medicine index, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BLACHERE, N.E. et al. Heat shock protein-based cancer vaccines and related thoughts on immunogenicity of human tumors. Cancer Boilogy. December 1995, Vol. 6, No. 6, pages 349-355, see entire document.	1-39
A	HEIKE, M. et al. Heat shock protein-peptide complexes for use in vaccines. J. Leuk. Biol. August 1996, Vol. 60, pages 153-158, see entire document.	1-39

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
10 JUNE 1999

Date of mailing of the international search report
07 JUL 1999

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06048

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/06048

∴. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 39/395 45/00, 45/05; A01N 37/18, 43/04, 63/00; C12N 5/02, 5/06, 5/08; C07K 1/00, 16/46; C07H 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/130.1, 138.1, 183.1, 278.1, 93.71; 435/ 325, 355, 372.3, 373; 514/2, 44; 530/350, 391.7; 536/23.1, 23.2, 23.4, 23.5

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-4, 14-17, 38 and 39, drawn to a method for inducing an antigen-specific immune response in a subject by in vivo administration of an antigenic peptide binding protein and a cytotoxic agent.

Group II, claim(s) 5-13, 18-30, 38 and 39, drawn to in vivo administration of polynucleotides which encode an antigenic peptide binding protein and encode a cytotoxic agent.

Group III, claim(s) 31-39, drawn to in vivo treatment with effector cells which have been primed in vitro with an antigenic peptide binding protein and a cytotoxic agent.

The inventions listed as Groups I-III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The special technical feature which links the three groups is the combination of an antigenic peptide binding protein and a cytotoxic agent. One embodiment of such an antigenic peptide binding protein would be a monoclonal antibody. The linking of monoclonal antibobies to cytotoxic agents in order to specifically target a particular antigen and kill the cell bearing said antigen has been well known in the art for many years.

The special technical feature of Group I is considered to be treatment of a subject using an antigenic peptide binding protein and a cytotoxic agent directly administered to the subject to effect an immunospecific response.

The special technical feature of Group II is considered to be treatment of a subject using nucleic acid sequences which encode an antigenic peptide binding protein and a cytotoxic agent which are translated in vivo to effect an immunospecific response.

The special technical feature of Group III is considered to be the in vitro priming of immune effector cells with an antigenic peptide binding protein and a cytotoxic agent and treatment of a subject with the primed effector cells in order to exert an immunospecific response in the subject.

Accordingly, Groups I-III are not so linked by the same or a corresponding special technical feature as to form a single general inventive concept.